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The mitochondrial 60-kDa heat shock protein in marine invertebrates: biochemical purification and molecular characterization

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Abstract Sessile marine invertebrates undergo constant direct exposure to the surrounding environmental conditions, including local and global environmental fluctuations that may lead to fatal protein damage. Induction of heat shock proteins (Hsps) constitutes an important defense mechanism that protects these organisms from deleterious stress conditions. In a previous study, we reported the immunological detection of a 60-kDa Hsp (Hsp60) in the sea anemone *Anemonia viridis* (formerly called *Anemonia sulcata*) and studied its expression under a variety of stress conditions. In the present study, we show that the sponge *Tetilla* sp. from tidal habitats with a highly variable temperature regime is characterized by an increased level of Hsp60. Moreover, we show the expression of Hsp60 in various species among Porifera and Cnidaria, suggesting a general importance of this protein among marine invertebrates. We further cloned the *hsp60* gene from *A viridis*, using a combination of conventional protein isolation methods and screening of a complementary deoxyribonucleic acid library by polymerase chain reaction. The cloned sequence (1764 bp) encodes for a protein of 62.8 kDa (588 amino acids). The 62.8-kDa protein, which contains an amino terminal extension that may serve as a mitochondrial targeting signal, shares a significant identity with mitochondrial Hsp60s from several animals but less identity with Hsp60s from either bacteria or plants.

INTRODUCTION

It is well established that organisms in nature have the ability to express heat shock proteins (Hsps) in diverse patterns in response to stressful stimuli (Lindquist 1986; Morimoto 1993; Welch 1993; Hofmann 1999). The observed variation includes expression of different isoforms within a family of Hsps, variations in endogenous level of Hsps, changes in the kinetics of Hsp expression, and changes in the thresholds at which *hsp* genes are activated (Feder 1999). Evolution of this plasticity and the response of transcriptional activation of *hsp* genes to changes in environmental temperature are critical yet largely unexplored questions in the ecological physiology of heat shock response (Hofmann 1999).

To understand the mechanisms behind the plasticity of the 60-kDa Hsp (Hsp60) expression and its function in adaptation mechanisms in nature, it is necessary to study the biochemical and molecular nature of Hsp60 in organisms that are exposed to stress in their natural habitat. This argument is particularly relevant in certain groups of sessile marine invertebrates such as sponges and cnidarians, including reef-building corals that form communities of unique ecological importance. Being sessile organisms, they experience constant direct exposure to the surrounding environmental conditions and are exposed to local and global environmental fluctuations that may lead to a cellular protein damage and subsequent death (Brown 1997; Feder and Hofmann 1999). It is therefore interesting to elucidate the probable responses of these organisms to the predicted environmental challeng-

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es and to assess how these might affect their physiology and tolerance, distribution, and population dynamics (Gates and Edmunds 1999). One of the most intriguing habitats in nature is that of the rocky intertidal zone, which is characterized by steep gradients in environmental factors such as temperature, ultraviolet (UV) radiation, and salinity (Hofmann et al 2002; Tomanek and Helmuth 2002). Thus, it was shown that intertidal mussel, *Mytilus trossulus*, undergo irreversible protein damage on exposure to temperature fluctuations on a seasonal basis (Hofmann and Somero 1995, 1996). Sessile organisms such as cnidarians and sponges, which inhabit several niches in the intertidal zone and lack any developed physiological regulatory systems, are expected to possess well-developed cellular adaptation abilities (Brown 1997). Hsps are known to be induced by various stresses (eg, extreme temperature, UV, and salinity changes), and it is suggested that they play a significant role in conferring tolerance to harsh conditions on these organisms (Lindquist 1986; Trautinger 2001; Spees et al 2002). Given the suggested protective function, a detailed study of Hsp60 in sessile marine invertebrates is of high interest to understand the capability of these animals to survive both local and global environmental changes. Choresh et al (2001) studied the expression of Hsp60 in response to thermal stress in the sea anemone *Anemonia viridis* (formerly called *Anemonia sulcata*) from an intertidal habitat. They found that *A viridis* induces Hsp60 expression in response to environmental stress. In the present study, we examined the expression of Hsp60 in several taxa of marine invertebrates and the effects of stressful environmental conditions on Hsp60 expression in the Mediterranean sponge *Tetilla* sp. from tidal pools. Furthermore, we cloned for the first time the entire *hsp60* gene from *A viridis* and analyzed its primary sequence in comparison with Hsp60 sequences from other organisms.

MATERIALS AND METHODS

Seawater temperature measurements

Seawater temperature was measured every 1 hour for periods of several days before and after seasonal collections of specimens and during the collection, by underwater electronic sensors (Optic StowAway, Cape Cod, MA, USA) that were firmly secured in tidal pools and in the subtidal zone (30-m depth). Temperature data were processed by LogBook for Windows version 2.04.

Collection and maintenance of animals

Fragments of scleractinian corals, hydrozoans, and Ascidia sp. were collected from degraded coral reef habitats in Eilat (northern Red Sea, Israel). Fragments of sponges, hydrozoans, scyphozoans, and anthozoans were collected from tidal pools and intertidal zones of the Israeli Mediterranean coast (between 31°40′N, 34°32′E and 33°05′N, 35°06′E). Specimens were immediately frozen in a dry-ice container on collection. *Tetilla* sp. specimens were concurrently collected from tidal pools during an extreme low tide (December 1998) and from 30-m depth at a distance of 2 km from the tidal pool. Specimens were immediately frozen as described above.

Invertebrate protein extraction

Approximately 2 g of animal tissue was removed from frozen coral skeleton or cut from other organisms using a scalpel. Tissue was frozen in liquid nitrogen and ground using a mortar and pestle. The tissue powder was suspended in 1 mL buffer containing 0.5 M NaCl, 100 mM Tris-HCl, pH 7.5, 10 mM ethylenediaminetetraacetic acid (EDTA) (Bythell et al 1995), and a cocktail of protease inhibitors consisting of 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1.4 μM *trans*-epoxysuccinyl-L-leucylamido-(4-guanido)butane (E-64), 130 μM bestatin, 1 μM leupeptin, 0.3 μM aprotinin, and 1 mM sodium EDTA (Sigma, Rehovot, Israel). Homogenates were centrifuged at full speed for 20 minutes at 4°C (Eppendorf microcentrifuge). Zooxanthellae were removed from the homogenates during the centrifugation step (Bythell et al 1995). Supernatants were frozen at −20°C.

Preparation of total soluble proteins for protein purification from *A viridis*

Homogenates of *A viridis* were prepared as described above in buffer containing 50 mM K^+ *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid (HEPES), pH 7.4, 10 mM $MgCl_2$, 100 mM NaCl, and 2 mM of the protease inhibitor phenylmethane sulfonyl fluoride. Homogenates were centrifuged at maximal speed for 20 minutes at 4°C (Eppendorf microcentrifuge). Supernatants were collected and recentrifuged in a Beckman ultracentrifuge at 30 000 rpm for 1 hour at 4°C. Supernatants were then collected, and buffer was exchanged to buffer A (50 mM K^+ HEPES, pH 7.4, 10 mM $MgCl_2$) using PD-10 column (Pharmacia, Rehovot, Israel).

Partial purification of Hsp60

A viridis protein solutions were loaded onto an ion-exchange chromatography column (12 mL, Source ISQ, Pharmacia) previously equilibrated with buffer A (flow rate 3 mL/min). At least 100 mg of total protein was loaded per run. The column was washed with 3 column volumes of buffer A. Proteins were eluted at a flow rate of 1 mL/min by a volume of 140 mL buffer A with a 0–1 M

Table 1 Description of PCR reactions[a]

PCR reaction	Primers	Template	Annealing temperature (°C)	Comments
1	A53, T7	cDNA	53	The PCR product is 6C
2	E35, DA53	6C	48	
3	Bfor, T7	6C	55	
4	Arev, T3	cDNA	55	The PCR product is 2CC
5	Arev, A53	2CC	50	
6	NA531rev, A1308	2CC	48	
7	D900rev, A1308	2CC	48	
8	G700rev, A1308	2CC	48	
9	K600rev, A1308	2CC	48	
10	FINAL5, T3	2CC	57	
11	MYR, T7	cDNA	54	

[a] PCR, polymerase chain reaction; cDNA, complementary deoxyribonucleic acid.

gradient of NaCl. Fractions of 4 mL were collected. The presence of Hsp60 was checked in every fraction by Western blot analysis, using a monoclonal antibody against Hsp60. Fractions containing Hsp60 were pooled and concentrated to a total volume of 1 mL using ultrafiltration tubes (Centricon). Subsequently, the samples were loaded onto a gel filtration column (Superdex 200, Pharmacia). The Biologic HR chromatography system (Bio-Rad, Rishon Le-Zion, Israel) was used for all chromatography steps at 4°C. Again, the collected fractions were screened for the presence of Hsp60 by Western blot analysis. Positive fractions (which contained Hsp60) were combined, concentrated, and loaded onto 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and immunoblotting

Aliquots of protein samples (10 μL) were used for protein concentration estimations according to Bradford (1976). Protein separation using SDS-PAGE and Western blots was carried out as described by Choresh et al (2001). Gels were silver stained or were blotted with a 1:1000 dilution of monoclonal antibodies raised against mitochondrial Hsp60 (mt-Hsp60) (IgG mouse clone LK-2, Sigma). Controls for specificity of the primary antibody were conducted using recombinant, purified Chinese hamster Hsp60 (Levi-Rimler et al 2001). Detection was performed with a secondary antibody (1:5000 dilution), anti-mouse IgG conjugated to horseradish peroxidase (Sigma). Bands were detected by enhanced chemiluminescence procedure (Harlow and Lane 1988). Densities of Hsp60 bands obtained in Western blot analysis were quantified using ImageMaster 1D densitometer. Intensities of the Hsp60 bands were normalized to the intensity of the reference protein band.

Protein sequence analysis

This procedure was carried out at the Smoler Protein Center, Technion (Israel Institute of Technology), Israel. The stained protein bands in the gel were cut with a clean razor blade, and the proteins in the gel were reduced with 10 mM dithiothreitol and modified with 100 mM iodoacetamide in 10 mM ammonium bicarbonate. They were then treated with 50% acetonitrile in 10 mM ammonium bicarbonate to remove the stain from the proteins, followed by drying the gel pieces. The dried gel pieces were rehydrated with 10 mM ammonium bicarbonate containing about 0.1 μg trypsin per sample. The gel pieces were incubated overnight at 37°C, and the resulting peptides were recovered with 60% acetonitrile with 0.1% trifluorocetate. The tryptic peptides were resolved by reverse-phase chromatography on 0.1× 300-mm fused silica capillaries (J&W, Palo Alto, CA, USA, 100 μm ID) home-filled with porous R2 (Perseptive, Framingham, MA, USA). The peptides were eluted using an 80-minute linear gradient of 5–95% acetonitrile with 0.1% acetic acid in water at flow rate of about 1 mL/min. The liquid from the column was electrosprayed into an ion-trap mass spectrometer (LCQ, Finnigan, San Jose, CA, USA). Mass spectrometry (MS) was performed in the positive ion mode using repetitively full MS scan, followed by collision-induced dissociation (CID) of the most dominant ion selected from the first MS scan. The MS data were compared with simulated proteolysis and CID of the proteins in the NR-NCBI database using the SEQUEST software (J. Eng and J. Yates, [University of Washington] and Finnigan, San Jose, CA, USA). Protein sequence comparisons were carried out using NCBI-BLASTP (Altschul et al 1990).

Cloning of Hsp60-encoding deoxyribonucleic acid

A complementary deoxyribonucleic acid (cDNA) library was constructed using ZAP Express® cDNA synthesis kit and ZAP Express® cDNA Gigapack® III Gold Cloning kit as described by Wiedenmann et al (2000). *A viridis hsp60* gene was cloned using several steps of nested polymerase chain reaction (PCR) as described in Tables 1 and 2. PCR was carried out in a thermal cycler (iCycler, Bio-Rad). The

Table 2 Primer details[a]

Primer	Sequence	Direction	Comments
E35	5′ TCYTCYTTIGGDATYTCIGT 3′	3′ → 5′	Based on *hsp60* genes alignment
DA53	5′ CCIACIAARGTIGTIMGIACIGC 3′	5′ → 3′	Based on *hsp60* genes alignment
Arev	5′ GGCAACTCCTGCAGCATCTG 3′	3′ → 5′	Based on E35-DA53 PCR product
Bfor	5′ CAGATGCTGCAGGAGTTGCC 3′	5′ → 3′	Based on E35-DA53 PCR product
A53	5′ GAYGCIYTIAAYGCIMGIGC 3′	5′ → 3′	Based on *hsp60* genes alignment
NA531rev	5′ GCGTCGCATTCAACGCGTC 3′	3′ → 5′	Based on A53-Arev PCR product
A1308	5′ AAYAAYACIAAYGARGARGC 3′	5′ → 3′	Based on sequence of purified HSP60 from *Anemonia viridis*
D900rev	5′ RAAICCIGGIGCYTTIACIGC 3′	3′ → 5′	Based on *hsp60* genes alignment
G700rev	5′ CCICKRTCRAAYTTCATICC 3′	3′ → 5′	Based on *hsp60* genes alignment
K600rev	5′ CCRTCYTTIACIGTDATIACICC 3′	3′ → 5′	Based on *hsp60* genes alignment
FINAL5	5′ GCCAGTACAGTCGCTGTTGT 3′	3′ → 5′	Based on A1308-NA531rev PCR product
MYR	5′ ATGTACCGATTACCAAGTTTAATT 3′	5′ → 3′	Based on FINAL5-T3 PCR product
T7	5′ GTAATACGACTCACTATAGG 3′	3′ → 5′	Vector primer
T3	5′ CGGAATTAACCCTCACTAAAG 3′	5′ → 3′	Vector primer

[a] PCR, polymerase chain reaction.

Table 3 Expression of HSP60 in marine invertebrates[a]

Phylum	Class	Species	Site of collection
Porifera	Demospongiae	*Chondrilla nucula*	Mediterranean
		Chondrosia reniformis	Mediterranean
		Spirstrella cuntatrix	Mediterranean
		Tetilla sp.	Mediterranean
		Cliona sp.	Gulf of Eilat (Red Sea)
Cnidaria	Hydrozoa	*Halocordyle distichia*	Mediterranean
		Aglaophenia pluma	Mediterranean
		Millepora dichotoma	Gulf of Eilat (Red Sea)
	Scyphozoa	*Cotylorhiza fuberculata*	Mediterranean
		Rhopilema nomadica	Mediterranean
	Anthozoa	*Anemonia viridis*	Mediterranean
	—Scleractinian corals	—*Oculina patagonica*	Mediterranean
		—*Stylophora pistillata*	Gulf of Eilat (Red Sea)
		—*Acropora variabilis*	Gulf of Eilat (Red Sea)
		—*Acropora eurystoma*	Gulf of Eilat (Red Sea)
		—*Acropora hemprichii*	Gulf of Eilat (Red Sea)
		—*Cyphastrea* sp.	Gulf of Eilat (Red Sea)
		—*Pocillopora danae*	Gulf of Eilat (Red Sea)
		—*Turbinaria* sp.	Gulf of Eilat (Red Sea)
		—*Favia favus*	Gulf of Eilat (Red Sea)
		—*Fungia granulosa*	Gulf of Eilat (Red Sea)
		Heteroxenia fuscescens	Gulf of Eilat (Red Sea)
Chordata		*Ascidia* sp.	Gulf of Eilat (Red Sea)

[a] HSP, heat shock protein.

reactions were performed in a volume of 50 μL PCR solution containing 1 μL of template (cDNA library or purified PCR product, Table 1); 10 pmol of each regular primer, or 100 pmol of each degenerated primer (Table 2); 0.2 mM deoxynucleoside triphosphates mix (Sigma); 2 mM $MgCl_2$; and 1.25 units of *Taq* polymerase (Dynazyme). The thermal cycler was programmed to 95°C (5 minutes) followed by 35 cycles of 95°C (30 seconds), annealing temperature according to Table 1 (30 seconds), and 72°C (60 seconds) followed by 72°C (6 minutes). The resulting products were visualized on 1% Tris-acetate-EDTA agarose gel, cleaned from the gel (NucleoSpin®, Macherey-Nagel), cloned into a pGEM-T-easy vector (Promega, Madison, WI, USA), and sequenced. Sequence analysis was carried out using NCBI-BLASTX (Altschul et al 1990).

RESULTS

Immunological evidence for the expression of Hsp60 in marine invertebrates

To examine the generality of expression of Hsp60, we studied the ability of various marine invertebrates to express this protein. Expression of Hsp60 was immunologically detected, for the first time, in representatives of Porifera (5 species), Cnidaria (16 species), and 1 Ascidia species (Chordata). The species are listed in Table 3. Fur-

thermore, to examine Hsp60 levels in the sponge *Tetilla* sp from different habitats, samples were taken from tidal pools and from a second habitat at 30-m depth. The tidal pools were subjected to strong fluctuations in seawater temperature over the sampling period. In contrast, the second sampling site at 30-m depth showed almost constant temperatures, however, on a slightly higher level (Fig 1C). As shown in Figure 1 A,B, samples of the sponge *Tetilla* sp. collected from tidal pools are characterized by 2- to 3-fold higher levels of Hsp60 compared with specimens from the habitat at 30-m depth.

Purification of Hsp60 from the sea anemone *A viridis*

To obtain sequences from *A viridis* Hsp60 that could help to isolate the gene, we decided to purify the protein from the animal. Partially purified protein was obtained by a 2-step purification using anion exchange (Hsp60 was eluted between 0.25 and 0.35 M of NaCl), followed by gel-filtration chromatography (elution volume at 51 mL), as described in the Materials and Methods. Fractions containing Hsp60 were pooled and concentrated for further analysis. As shown in Figure 2A, a protein band with a molecular weight of 61 kDa was relatively enriched after the final purification steps. This band was identified, using Western blot, as an Hsp60-related protein (Fig 2B). The Hsp60 band was cut from the gel and subjected to sequencing. Sequence data were obtained for 1 peptide of the purified protein (Fig 3). The amino acid sequence (25 amino acids long) was identical to amino acid residues of the mt-Hsp60 polypeptide of several organisms, including *Drosophila melanogaster*, *Paracentrotus lividus*, and *Mus musculus*, which shows the protein to be highly conserved between organisms, and suggests that this protein functions in the mitochondria. Analysis also revealed this fragment to be less similar to plastid and bacterial chaperonin-60.

Cloning of Hsp60-encoding DNA

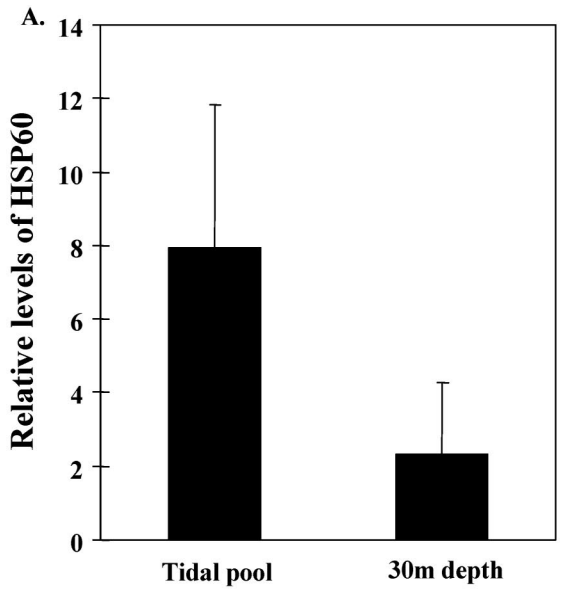
The obtained peptide sequence helped us to clone the entire *hsp60* gene from *A viridis* as follows. First, a forward degenerated primer (A1308, Table 2) was constructed on the basis of the amino acid sequence of this peptide. Second, degenerated reverse primers were constructed on the basis of an alignment of a conserved region of Hsp60 genes (Table 2). In the latter alignment, we included only Hsp60 sequences from organisms that showed full identity with the peptide sequence obtained from *A viridis* Hsp60. As shown in Figure 4, the cloned sequence (1764 bp) encodes for a protein of 588 amino acids (62.8 kDa, PI 5.3) and contains a classical mt-Hsp60 signature (amino acids 439–450, according to: http://www.expasy.org/prosite/; PROSITE: PDOC00268). Alignment of the deduced amino acid sequence of *A viridis* Hsp60 with Hsp60s from several eukaryotes and prokaryotes revealed that the highest scoring amino acid identities were to mt-Hsp60s from the sea urchin, *P lividus* (74.7%), the house mouse, *M musculus* (73.2%), the fruit fly, *D melanogaster* (73.2%), *Homo sapiens* (72.8%), and the nematode *Caenorhabditis elegans* (68.7%). It shared less identity with Hsp60s from bacteria (*Escherichia coli*, 50.9%), yeast (*Saccharomyces cerevisiae*, 54.4%), or plants (*Arabidopsis*, 56.9%). Alignment also revealed that the protein contains a 28-amino acid extension at the N terminus, which may serve as a mitochondrial targeting signal (Fig 4) (Emanuelsson et al 2001).

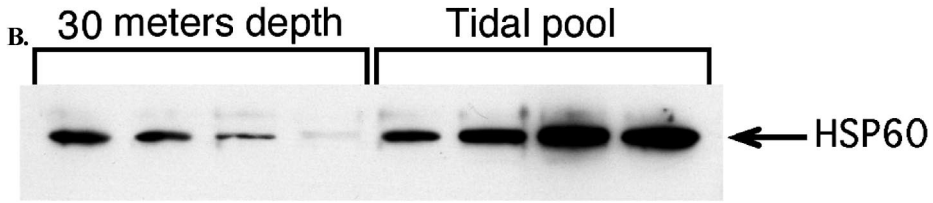
Sequence analysis of the mature mt-Hsp60 protein

Given that the mitochondrial presequence is cleaved after amino acid 28 (phenylalanine), the mature protein is 560 amino acids long, with a calculated molecular weight of 59.7 kDa and a PI of 5.06. As expected, the mitochondrial presequence is less conserved among the various species, and it contains a majority of nonpolar amino acids (57% of the sequence), 18% of basic acids (arginine), and no acidic amino acids (Bedwell et al 1989; Emanuelsson et al 2001). As also typical to mt-Hsp60s, the protein has a conserved GGM repeats at its C-terminal end. The structure and function of this region are not known (Gupta 1995; Sanchez et al 1999).

Molecular chaperones have an essential function in promoting the adenosine triphosphate (ATP)–dependent folding of proteins under both normal and stress conditions (Hemmingsen et al 1988; Goloubinoff et al 1989; Hartl 1996; Bukau and Horwich 1998). The ATP binding site is 1 of the most conserved regions of the protein (Brocchieri and Karlin 2000). As shown in Figure 4, the sea anemone Hsp60 contains an ATP-binding motif at amino acids 201–222, which also has a higher identity to ATP binding sites from vertebrates than from the invertebrate C elegans (Fig 5A).

The bacterial Hsp60 (GroEL) is a tetradecamer, cylindrically shaped, that is organized in 2 heptameric rings stacked back to back (reviewed by Sigler et al 1998). The double-ring structure of GroEL is stabilized by a number of intersubunit contacts including a salt bridge between the glutamic acid in position 461 (E461) and arginine in position 452 (R452) (Brocchieri and Karlin 2000). Despite a high sequence homology to the bacterial chaperonins, the mammalian mt-Hsp60 forms heptameric single-ringed structures in the absence of nucleotides and Hsp10 (Viitanen 1992; Levi-Rimler et al 2002). The dissociation of the double rings into single rings is attributed to the fact that the salt bridge is absent in mammalian mt-Hsp60 (methionine is present in position 449, which corresponds to arginine R452 in bacteria). Interestingly, *A*





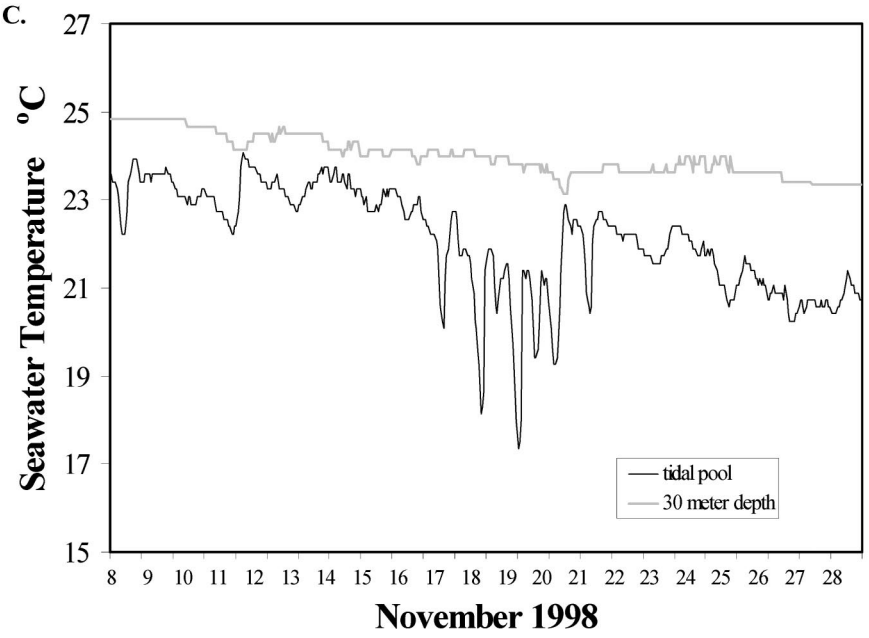


Fig 1. (A) Expression of Hsp60 in the Mediterranean sponge *Tetilla* sp. Comparison between individuals collected from a stressful habitat (tidal pool) and individuals collected from a benign habitat (30-m depth) ($n = 4$, Kruskall-Wallis, $P < 0.05$). (B) Western blot analysis showing the differential expression of Hsp60 in sponges from tidal pools and from 30-m depth. Each band represents 1 specimen. (C) Seawater temperature fluctuations in a tidal pool and in 30-m depth during the experiment. Specimens were sampled simultaneously from a tidal pool and from 30-m depth at the same area. The average seawater temperature (temperature was measured every hour, $n = 24$) in the tidal pool during the sampling day (19 November 1998) was 20.69 ± 1.32°C, whereas the average seawater temperature in 30-m depth during the same period of time was 23.98 ± 0.008°C ($n = 24$). Hsp, heat shock protein.

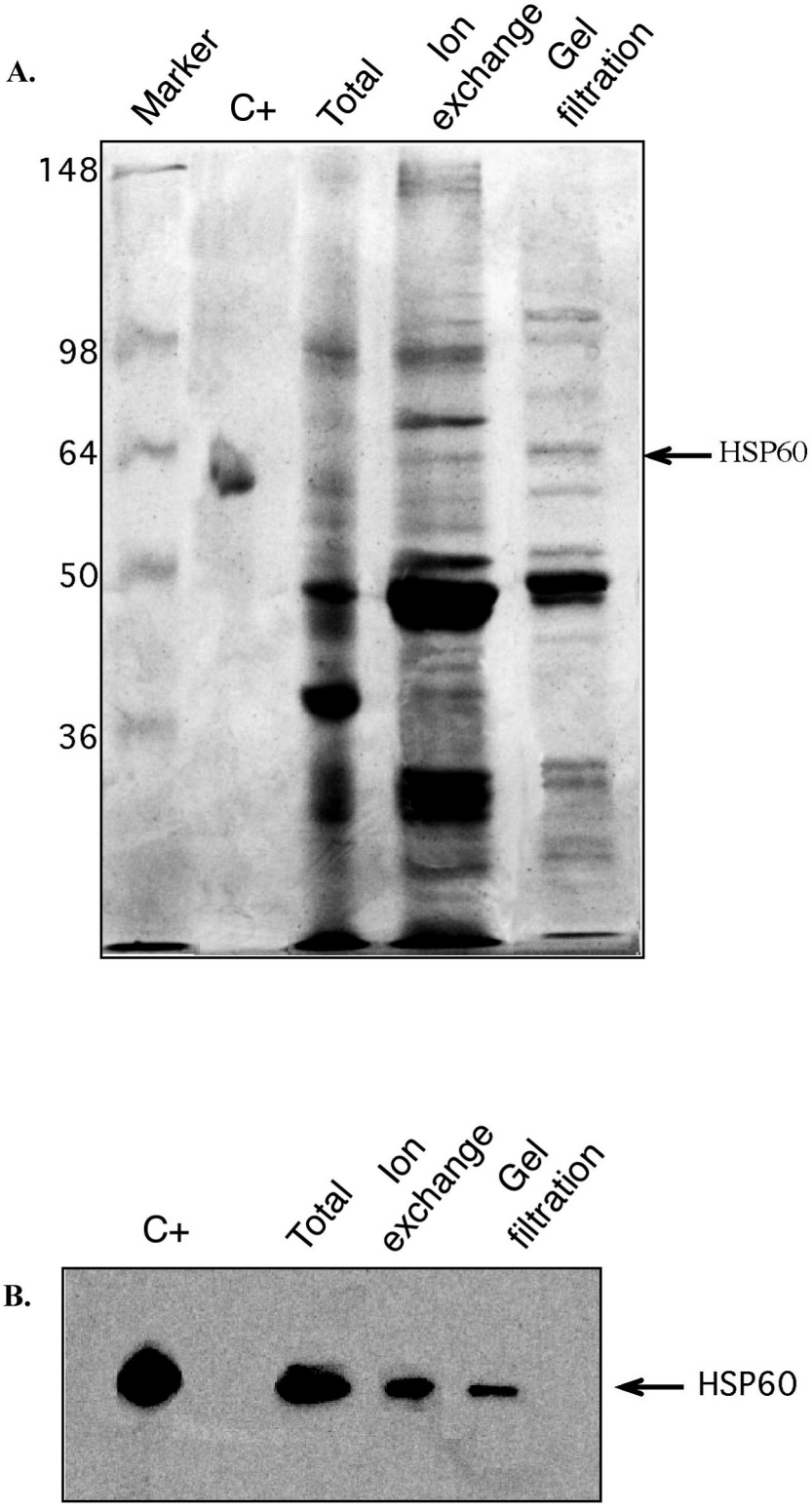


Fig 2. (A) Silver-stained SDS-PAGE of protein fractions during the purification process. Marker: molecular weight marker (kDa). C+: positive control (purified Chinese hamster mt-Hsp60). Total: total protein extraction after desalting. Ion exchange: pooled and concentrated fractions, immunologically reactive with anti-Hsp60 antibodies, after anion exchange chromatography. Gel filtration: concentrated fractions, immunologically reactive with anti-Hsp60 antibodies, after gel filtration chromatography. A distinct band with molecular weight of 61 kDa can be seen in ion-exchange and gel-filtration fractions. This band was found to be Hsp60 and was extracted from the gel for further sequencing. (B) Immunological comparison of Hsp60. C+: positive control (purified Chinese hamster mt-Hsp60). Total: total protein extraction after desalting. Ion exchange: pooled and concentrated fractions, immunologically reactive with anti-Hsp60 antibodies, after anion-exchange chromatography. Gel filtration: concentrated fractions, immunologically reactive with anti-Hsp60 antibodies, after gel filtration chromatography. SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; mt-Hsp60, mitochondrial heat shock protein 60.

viridis Hsp60 contains histidine at position 458 (corresponding to R452 in E coli). Histidine is known to create a salt bridge at some pH conditions (Katz 1997), placing the oligomeric structure of Hsp60 from *A viridis* in the middle, between the bacterial chaperonins (absolute double-ring structure) and the mammalian mt-Hsp60 (absolute single-ring structure when tested using gel filtration in the absence of nucleotides).

Fig 3. Comparison of partial amino acid sequence of the mt-Hsp60 from *Anemonia viridis* with amino acid sequences of other organisms (BLASTP). Organisms that show full identity are underlined. Gray indicates amino acids identity for all sequences.

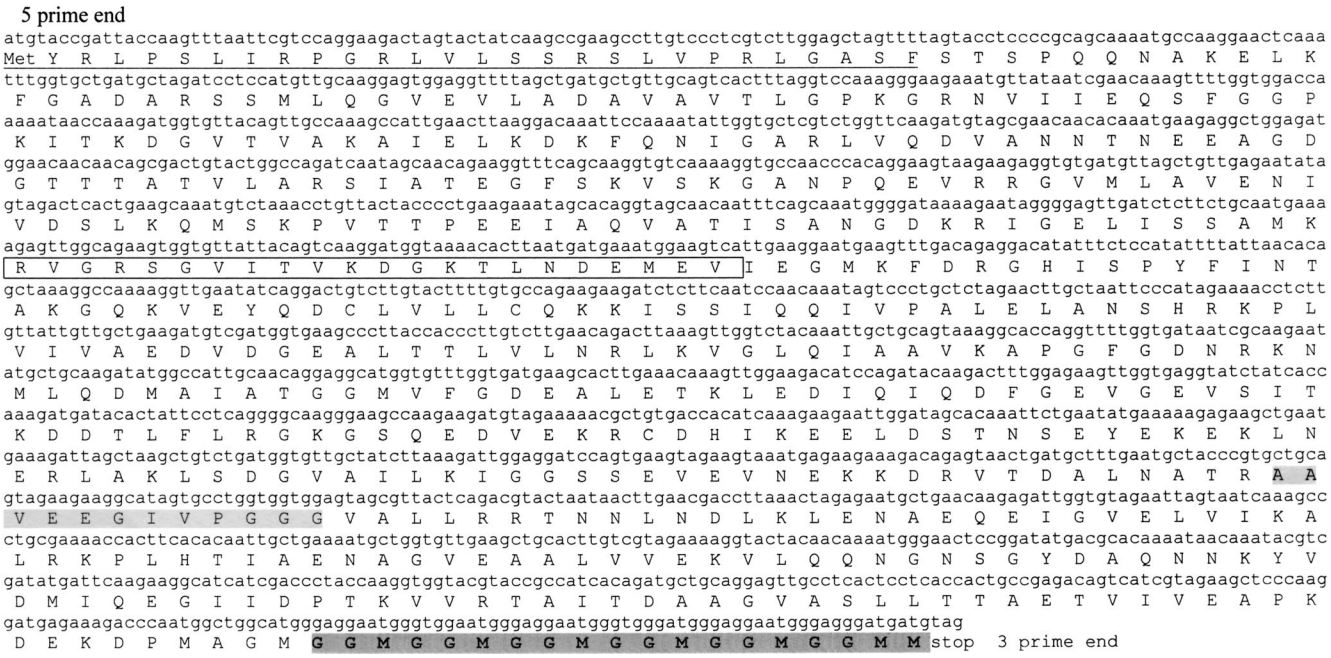


Fig 4. Nucleotide and amino acid sequence of the cloned Hsp60 from *Anemonia viridis* (Accession number AY500892). The 1764-bp (588 amino acids) sequence contains a presequence of 28 amino acids at the N terminus that is required for import into the mitochondria (underlined), a classical mt-Hsp60 signature (marked by light gray), an ATP-binding motif (boxed), and a typical GGM repeat motif at the C terminus (marked by dark gray). Hsp, heat shock protein; mt-Hsp60, mitochondrial Hsp60; ATP, adenosine triphosphate.

DISCUSSION

Hsp60 is known to function as a molecular chaperone in many species (Lindquist 1986; Hemmingsen et al 1988; Goloubinoff et al 1989; Hartl 1996) and is absolutely essential for the proper functioning of cells under normal and stress conditions. Nevertheless, little is known about the physiological relevance of Hsp60 in the environmental stress response of marine invertebrates. Our previous study revealed the expression of this protein in the sea anemone, *A viridis,* and the effect of extreme changes in seawater temperature on its expression (Choresh et al 2001). Other studies have revealed a correlation between increased stress, related to higher seawater temperatures, and an upregulation of Hsp60 in the scleractinian corals *Montasraea faveolata* and *Montastraea annularis* (Downs et al 2000, 2002).

To further elucidate the involvement of Hsp60 in the adaptation of sessile marine invertebrates to environmental stress, we examined the ability of various species to express this protein. Induction of Hsp60 expression, as examined by Western blot, was found in 23 species of Porifera, Cnidaria (including scleractinian corals), and

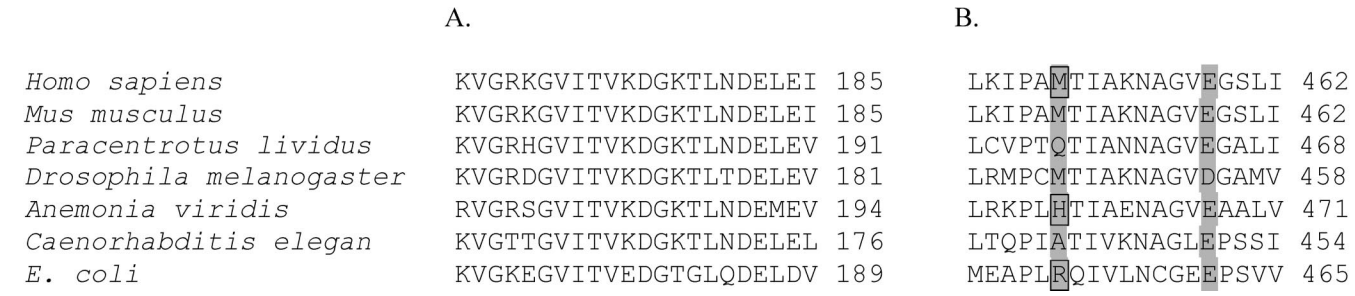


Fig 5. Comparison (alignment) of parts of the mt-Hsp60 from several organisms. (A) ATP-binding motif. *Anemonia viridis* shares 82% identity with ATP binding sites from *Drosophila melanogaster* and mammals. It shares less identity (77%) with ATP binding site from *Caenorhabditis elegans*. (B) Alignment of interaction region between Hsp60 rings. In bacterial Hsp60, interaction occurs between E461 (shaded) at first ring and the basic amino acid R458 at the second ring (shaded and boxed). Mammals, *C elegans*, and *D melanogaster* have amino acids (M, Q, or A, shaded and boxed) that do not interact to produce a salt bridge. *A viridis* posses H458 (shaded and boxed), which may produce a salt bridge at some pH conditions. mt-Hsp60, mitochondrial heat shock protein; ATP, adenosine triphosphate.

Chordata (Table 3). This finding indicates a general importance of Hsp60 for many marine invertebrates.

Mitochondria are essential eukaryotic organelles that serve as a site for many vital metabolic pathways and supply the cell with oxidative energy. Hsp60 plays a central role in the folding of newly imported and stress-denatured proteins (Martin et al 1992; Martinus et al 1995). As so, it was demonstrated that yeast containing mutated mt-Hsp60 do not grow at elevated temperatures (Cheng et al 1989; Dubaquie et al 1998) and show irreversible aggregation of a large number of newly imported proteins (Dubaquie et al 1998). The higher level of Hsp60 expression by sponges from stressed habitats such as tidal pools, compared with levels of Hsp60 expression by sponges from a benign habitat (30-m depth), strengthens the idea that this protein has a significant role in the adaptation of sessile marine invertebrates to highly fluctuating environmental conditions. However, this conclusion has to be addressed very carefully because tidal habitats are also characterized by increased UV or light stress or osmotic stress. These factors may also enhance the expression of Hsp60 (Sanders 1993).

As a first step toward the molecular characterization of Hsp60 from *A viridis,* we purified and partially sequenced the protein from the animal. Partial amino acid sequence determination revealed this protein to be similar to chaperonin-60 polypeptides from mitochondria of several animals (Fig 3). The fact that the obtained sequence exhibited only 50–70% identity to sequences from bacteria and plants indicates that the origin of the protein is from the animal rather than from the symbiotic zooxanthellae or associated bacteria.

Degenerative primers were designed on the basis of the sequence of the Hsp60 peptide and on nucleotide sequence comparison of known Hsp60 sequences from animals that showed full identity to the Hsp60 peptide. Indeed, after several steps of PCR, we were able to obtain the full sequence of mt-Hsp60 from *A viridis.*

It was shown previously that sponge proteins are more similar to those of mammals than to proteins of the invertebrate *C elegans* (Gamulin et al 2000). The latter observation was attributed to a slower evolutionary rate in deuterostomes, more specifically the chordate lineage (Gamulin et al 2000). Sequence analysis showed that Hsp60 of *A viridis* is more similar to vertebrate Hsp60s than to Hsp60 of the invertebrate *C elegans*. However, the observed difference is not statistically significant. More sea anemone proteins must be cloned and sequenced to show whether *A viridis* proteins are more similar to those of vertebrates, as was shown for sponges.

Most Hsp60s were purified as proteins composed of 14 subunits organized in 2 heptameric rings. In contrast, mammalian mt-Hsp60s have been purified as a single-ring molecule (Viitanen 1992; Levi-Rimler et al 2002). This structure was attributed to the fact that the salt bridge that formed between amino acids R542 and E461 in bacteria is absent in mammalian mt-Hsp60 (because of the replacement of R452 by M449). Interestingly, the *A viridis* mt-Hsp60 contains the amino acid histidine (Fig 5) at the corresponding position (H458 instead of R452 or M449). Thus, *A viridis* Hsp60 may form double-ring structures under certain pH conditions. Alignment shows that this amino acid occurs at this position only in the sea anemone, when compared with other eukaryotes (Fig 5B). Nevertheless, the elution volume of *A viridis* in gel filtration is similar to a single-ring Hsp60s (not shown). A study of the biochemical traits of this protein may reveal unusual properties that could have interesting consequences for stress adaptation.

In this study, we reported the first identification of mt-Hsp60 from Cnidaria at the molecular level. The results of this study represent an essential step toward understanding the role of Hsp60 in marine invertebrate adaptation, which may have implications for organism abundance and distribution in various habitats. There is a growing concern among scientists and coral reef man-

agers over the worldwide degradation of coral reefs as a result of global climate changes and human perturbations. Thus, making the development of an early warning system for assessment of the health of marine benthic communities is of great importance. Such a system should be based on accurate understanding of the responses of the organism to environmental changes. Characterization of Hsp responses in coral reef organisms may help to predict their ability to survive future short- and long-term changes in seawater temperature (Ryan and Hightower 1996).

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